

## Bacteria Present in the Intestinal Tract of *Melipona quadrifasciata anthidioides* Lepeletier (Hymenoptera, Apidae, Meliponinae)

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**Abstract.**—Scanning and transmission electron microscopy were used to study the microbial flora present in the gut of a Brazilian stingless bee, *Melipona quadrifasciata anthidioides* Lepeletier (Hymenoptera, Apidae). At least 5 bacterial morphotypes were found, but only the flora present in the hindgut maintain relationships with the food and the epithelial wall, indicating that it is autochthonous.

### INTRODUCTION

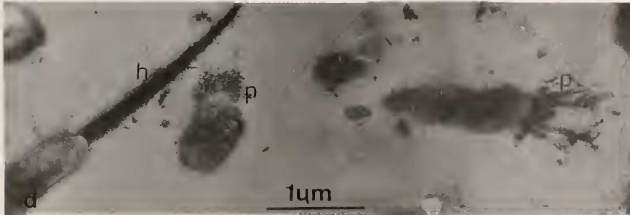
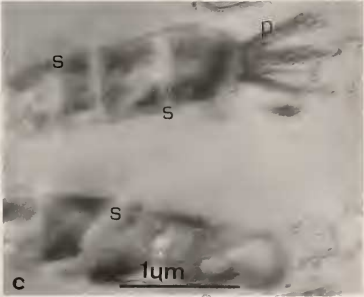
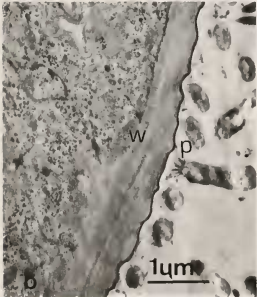
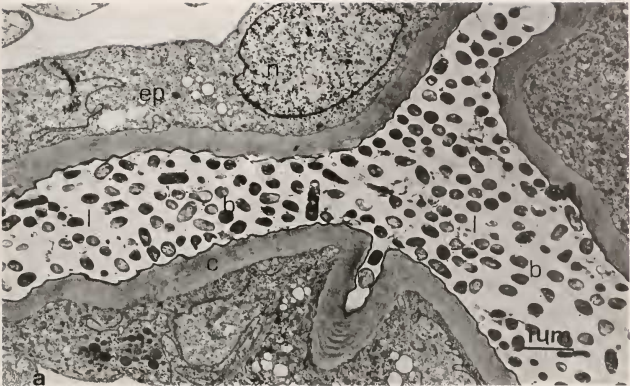
The association of microorganisms with the intestinal tracts of insects is varied and widespread (Buchner, 1965; McBee, 1977; Breznak and Pankratz, 1977; Bignell et al., 1980; Bignell, 1983; Cruz-Landim and Costa-Leonardo, 1995 ab; Oliveira et al. 1995). In bees, the presence of bacteria in the gut or association with the digestion of food, has been reported by several authors (White, 1921; Kluge, 1963; Trienko, 1965; Giordani and Scardovi, 1970; Machado, 1971; Cruz-Landim, 1972, 1990; Gilliam and Prest, 1987; Gilliam et al., 1988).

It has long been recognized that the gut microbiota plays a significant role in digestion. However, the reports of insect-microbe associations usually describe populations in the hindgut where the bulk of digestion has already been completed. Colonization of the midgut is much less common and is generally restricted to insects without a peritrophic membrane (Bignell et al., 1980; Caetano e Cruz-Landim, 1985).

Most microorganisms found in the insect gut exist freely in the lumen, but others attach themselves to the intima (Strambi and Zybberberg, 1967; Cruz-Landim, 1972; Fogelson et al., 1975; Breznak and Pankratz, 1977; Bracke et al., 1979; Bignell et al., 1979; Bayon, 1981; Caetano e Cruz-Landim, 1985). Bacterial attachment is often an essential initial step in colonization of host tissues and subsequent establishment of functional relationships. Attachment can be a highly specific process that involves fimbrial or nonfimbrial proteins on the outer membrane of the bacteria (Costerton et al., 1978; Hacker, 1992; Hoepelman and Tuomamen, 1992) and structural adaptations of the gut wall (Crawford et al., 1983).

In this paper light and electron microscopy were used to describe, for the first time, the intestinal microbial flora in *Melipona quadrifasciata anthidioides*, (Cruz-Landim, 1990) a Brazilian native stingless bee. The intent was determine the morphological diversity of the bacteria that

Fig. 1. TEM of bacteria in the crop of foragers of *M. q. anthidioides*: a, general view of the bacteria (b) in the crop lumen (l) (c= cuticle, ep= epithelium, n= nucleus); b, bacteria (b) presenting a tuft of pilli (p) directed toward the crop wall (w); c, cells showing division septa (s) and cross striated pilli (p); d, Aspects of the bacterial horns (h) formed by cross striated pilli (p) or fimbriae.



colonize the gut and whether the gut epithelium serves as a site for the attachment of these bacteria.

### MATERIAL AND METHODS

The descriptions in this study are based on guts of nurse workers of *Melipona quadrifasciata anthidioides* collected directly from colonies maintained in cages at the Biology Department, apiary in Rio Claro, SP, Brazil. The workers were allowed to fly freely in nature, and no additional food was given to them. The capture was done in the summer time. The material examination was done with transmission (TEM) and scanning (SEM) electron microscopy.

Guts to be observed by SEM were excised from the workers under buffered saline for insects, cut into anatomic parts, and fixed in Karnowsky (1965) during 2h at room temperature. The pieces were then freeze-fractured in liquid nitrogen, dehydrated in a graded ethanol series, critical point dried and covered with sputtered gold.

Dissected gut tracts were also prepared for TEM by fixation of the pieces in 2.5% glutaraldehyde in 0.1M cacodylate buffer during 2h at 4°C. Tissues were then rinsed in the buffer, post-fixed in 1% osmium tetroxide in the same buffer and dehydrated in a graded series of ethanol. The specimens, embedded in Epon-Araldite were thin-sectioned with glass knives and stained with uranyl acetate and lead citrate. Some additional preparations were done by emptying the gut parts on to coated grids and staining with 1% PTA (phosphotungstic acid) for negatively contrasted examination of the microorganisms.

Micrographs were taken with an Zeiss EM9S2 (TEM) and a P15 JEOL (SEM).

### RESULTS

Microorganisms were found in all parts of the worker bees alimentary canal. In the foregut bacteria were observed in the crop (Fig. 1) where they were distributed freely and homogeneously in the lumen. In the

sections, the cell profiles were mainly round shaped with, some rod-shaped ones among them (Fig. 1a). They are probably all bacillus, the round profiles being cross sections of the rods. The diameters of the rod and round cells are very similar, about 0.5  $\mu\text{m}$ . The greater incidence of round-shaped bacteria could be due to a preferential orientation of most cells in relation to the plane of the section.

Some cells have tufts of short pilli or fimbriae in one pole. In this case the pole provided with pilli is turned toward the crop wall (Fig. 1b). Other cells have "horns" apparently formed by the sticking together of long fimbriae (Fig. 1d). The "horns" show cross striations, and do not determine any special orientation of the cells. When the cells were observed in division (Fig. 1c), the cross trabeculae separate short compartments and the walls between them are thick (about 160 nm). Some cells have an inconspicuous, fuzzy capsule.

In the midgut the bacteria are mainly long rods (Fig. 2a, b) with the same diameter as the foregut cells (0.5  $\mu\text{m}$ ). However their distribution seems to be chaotic, and no fimbriae or "horns" were ever seen. Some cells have irregular contours, indicative of the presence of an undulating membrane (Fig. 2b). The bacteria in the midgut seem to concentrate in the anterior portion, near to the esophagic valve and posteriorly, near the pylorus.

The hindgut has the richest microorganism flora of the bee digestive tract. Bacteria are found in the ileum and in the rectum. In the ileum the bacteria adhere to some regions of the cuticle (Figs. 3, 4b). The bacterial population, formed mainly by long rods (3–4  $\mu\text{m}$  long  $\times$  0.4  $\mu\text{m}$  diameter) occupies almost all the ileum lumen, leaving free only the spaces filled by food particles (Fig. 4a, b). The bacteria tend to group around electron-dense material near the ileum wall (Fig. 4b). This electron-dense material when located at some distance from the wall appear as



Fig. 2. Bacteria in midgut of *M. g. anthidoides*: a, negative staining of a bacillum from midgut. m = microvilli; b, bacteria in the anterior portion of the midgut, showing at least two morphotypes (b 1 and b2).



Fig. 3. SEM of the hindgut cuticle of *M. q. anthidioides* (c) with adherent groups of bacteria b).

amorphous and irregular fragments, while close to the cuticle it appears formed by a fibrous material. Inside the cuticle it is possible to see some dots of electrondense content (Fig. 4b).

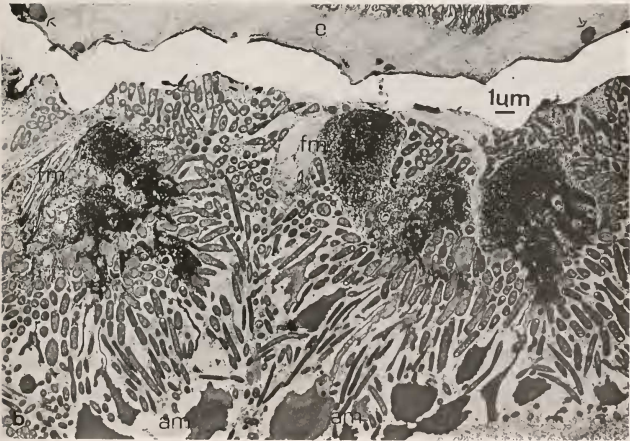
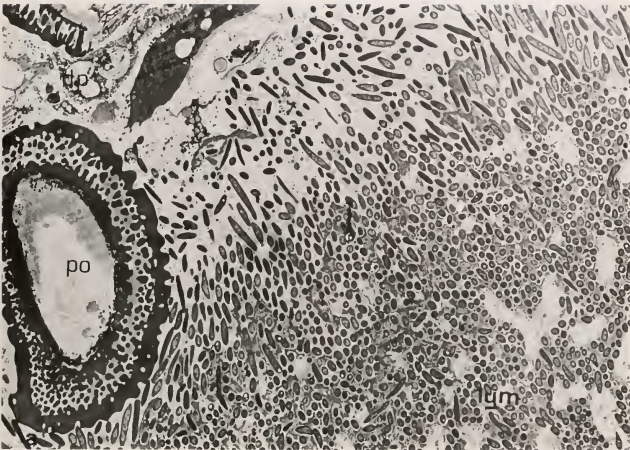
In the rectum, most bacteria are located over the rectal papillae (Fig. 5a, b), but are also attached to the rectum wall (Fig. 5a). They are rod shaped, measuring 3–4 μm long by 0.5 μm wide. The bacteria linked to the rectum wall have a tuft of pilli by which they attach themselves to the rectal wall. The attachment is not direct but through a thin layer of fuzzy material (Fig.

5c). The rods are straight and have a thick wall.

#### DISCUSSION AND CONCLUSIONS

The "in situ" examination of the microbiota of *Melipona quadrifasciata anthidioides* bee workers shows that only bacteria are present. Only a few different morphotypes were apparent. For instance, three in the foregut (bacilli without pilli, bacilli with pilli and bacilli with horns); two in the midgut (long straight bacilli and bacilli with an undulating membrane); three in the hindgut (long bacilli with pilli, bacilli

Fig. 4. TEM of bacteria in the hindgut of *M. q. anthidioides*: a, content of the hindgut showing the bacteria, an empty pollen grain (po) and digested food (dp); b, bacteria around amorphous material (am) near the hindgut lumen and fibrous (fm) material near the wall. The arrow points to electron-dense material inside the cuticle (c).





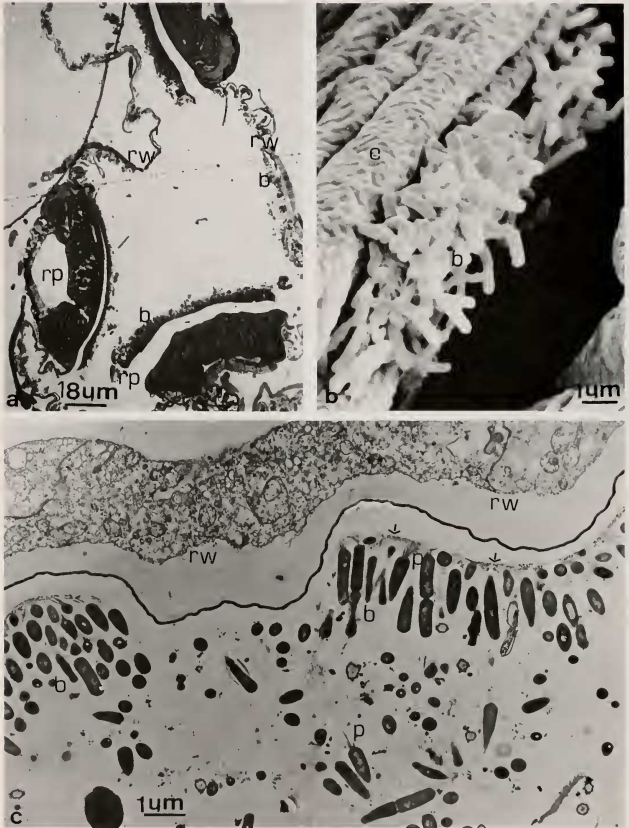


Fig. 5. Bacteria in the rectum of *M. q. anthidioides*: a, light microscopy of a thick section showing the rectum wall (rw) and the rectal papillae (rp) with masses of bacteria (b) over it; b, SEM of bacteria (b) attached to the rectum cuticle (c); c, TEM of the same region shown in b. The arrows point to a fuzzy material where bacteria (b) attach (p = pilli, rw = rectum wall).

without pilli and slim rods). Almost all animals have an autochthonous flora in the gut, formed by indigenous forms that colonize the individual early in its life, and remain throughout the life of the healthy animal (Savage, 1972).

*Melipona quadrifasciata* eats nectar and pollen. The pollen grain is difficult to digest because of its cellulose envelope. Studies by Machado (1971) and Gilliam et al. (1990) show the presence of bacteria in the pollen reserves in the colonies of this bee, where they are supposed to play a part in pre-digestion of pollen. However, a role in cellulose digestion is also attributed to the microorganisms present in the gut (Gilliam et al., 1988; Breznak and Brune, 1987).

The arrangement of the bacteria in the different parts of the gut may give some clues of their function. The bacteria maintaining special relationships with the gut wall, or with a special localization, may be autochthonous while the others may have been ingested with food. Bacteria attached to the foregut or midgut wall were rare or absent, so the bacteria found there may be in transit. In agreement with this interpretation is the fact that no special spacial or morphological relationships were observed between the bacteria and the food present in the midgut lumen.

In the ileum the bacteria group around what seems to be fragments of the pollen shell (Fig.4b). Close to the gut wall, this material seems to have undergone some transformation. It changes from a compact amorphous appearance to a fibrillar one, perhaps due to bacteria action. Some of this electron-dense material may cross the cuticle covering the ileum epithelium, since electron-dense spots may be seen inside the cuticle.

A great concentration of bacteria may be observed, parallel to the wall, or randomly distributed, over the rectal pads as has already been reported for *Apis mellifera* (Cruz-Landim, 1972). The bacteria in the rest of the rectum are perpendicular to

the wall and linked to it by pilli tufts. This special location seems to indicate particular functions of these bacteria, linked to bee physiology. The indications are that the microbial flora of the hindgut are autochthonous, or at least the parts close to the walls, or that maintain characteristic relationships with the wall or the food. However part of the bacterial flora in the bee gut is not autochthonous and may be digested or eliminated with the feces as seen in the honey bee by Gilliam and Prest (1987).

The physical intimacy of the autochthonous flora with the host probably reflects an underlying biochemical mechanism, such that the attachment of bacteria to the gut epithelium should afford a prime opportunity for nutrient exchange between the cells.

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